CALLUS AND ORGANIZED GROWTH

IN THE GRAMINEAE

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ABSTRACT

Linsmaier-Skoog's medium with coconut milk and 2,4-D (2,4-dichlorophenoxy acetic acid) was used to induce callus tissues from the inflorescences of 17 grass species and 3 grass varieties. The Linsmaier-Skoog's medium with only coconut milk was used for the development of callus tissues into organized growth.

The technique of using flasks and test tubes was mainly applied for the purpose of callus formation and organized growth from callus. The rotator and auxophyton techniques were also tried in the experiment.

An anatomical study was carried out by the paraffin method on callus tissues of one grass species and one grass variety, in which both formed callus tissues, but only the former formed plants from callus tissues.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF ILLUSTRATIONS

INTRODUCTION

Plant tissue culture in the form of callus growth was a relatively recently developed technique although the basic idea of the culture and continued growth of the individual cell and its relation to neighboring cells was pointed out by Haberlandt in 1902 (cited in Krikorian and Berquam, 1969).

The expectation that an individual cell possessed the capability of dividing to form tissues, then organs and then an entire plant has been investigated by various workers such as Gautheret (quoted in White, 1954), Steward (1958), Skoog (1944), White (1939), etc. This capacity of cells to form complete plants was known as "totipotency" and was first postulated for somatic cells under aseptic culture by Haberlandt.

Recently a method was developed to obtain callus tissues from the inflorescences of monocotyledonous plants and subsequently to obtain plants from these tissues (Heinz and Mee, 1969; Urata, unpublished).

The methods of Heinz and Mee (1969) and Urata (unpublished) were tested over a large number of grass species. Several types of media were used to determine the potential for differentiation from callus tissue to organized plant growth.

REVIEW OF LITERATURE

Early work on plant tissue culture

After Haberlandt formulated the idea of plant tissue culture at the beginning of the century (cited in Krikorian and Berquam, 1969), callus formation was attempted by many workers. The first step was made by Robbins (1922) with the root tips of peas, corn and cotton, and by Kotte (cited in White, 1954) with the stem tips of various species of higher plants. They reported limited growth of these materials. White (1934), on the other hand, reported the unlimited growth of tomato roots.

Tissue culture capable of unlimited growth and distinct from organ culture was first reported by White (1939), Gautheret and Nobecourt (cited in White, 1954). White used stem tissue from tobacco, and Gautheret and Nobecourt used cambial tissue from carrot roots and showed independently that callus tissues could be obtained from these materials and that they would grow indefinitely if they were repeatedly subcultured to fresh nutrient solutions. Since then different tissues of many species have been cultured and many investigations have also determined the optimum conditions for callus formation (Hildebrandt, Riker and Duggar, 1945, 1946; Caplin, 1947; Caplin and Steward, 1948; Caplin and Steward, 1949; Skoog and Tsui, 1948; Hildebrandt and Riker, 1953; Murashige and Skoog, 1962; Linsmaier and Skoog, 1965; etc.).

Aside from the dicots mentioned above, monocots had also been used as experimental material. Loo (1945) reported that callus which was formed from the excised stem tips of Asparagus officinalis L. had been maintained for nine months through twenty transfers. The imposed conditions were thus considered adequate for unlimited growth of this

material. This experiment was thought to be the first successful case of callus formation obtained from a monocotyledonous species.

Effects of various media on callus formation from different plant tissues

1. Media with auxin and cytokinin

Auxins and cytokinins are two of the three commonly recognized classes of growth hormones. Auxins include the indole derivatives such as indol-3-ylacetic acid, 2,4-D, naphthleneacetic acid. The naturally occurring cytokinins according to Letham (1969) are all purine derivatives, like zeatin, adenine, benzyl adenine and kinetin. Many successful attempts at callus formation from plants have been due to the use of these two growth hormones (Letham, 1969).

Steward and Caplin (1951) reported callus formation from the parenchyma cells of the potato tuber by supplementing the basal medium with coconut milk and 2,4-D. Coconut milk was considered as cytokinin since it had zeatin in it while 2,4-D was an auxin (Letham, 1969). Coconut milk and 2,4-D were extensively used as additives by a number of workers in later researches (Steward, Caplin and Millar, 1952; Steward and Shantz, 1955; Steward, 1958; Tomaoki and Ullstrup, 1958; Mitra and Steward, 1961; Steward, Mapes, Kent and Holsten, 1964; Heinz and Mee, 1969).

In grasses, Norstog (1956) reported a successful experiment of unlimited growth of Lolium perenne L. He obtained callus from endosperm on a medium containing White's solution, 2.0 percent sucrose, 1.0 mg/l indole-3-acetic acid, and 0.8 percent agar, as well as filtered coconut milk in amount of 20 percent of the total volume and 0.25 percent

yeast extract.

Carew and Schwarting (1958) obtained callus from rye embryo by using a modified Gautheret's medium containing sucrose, yeast extract, 2,4-D and casein hydrolysate. Ward (1960) obtained callus tissues from the mosses, Polytrichum and Atrichum using Knudson's medium supplemented with sucrose. He also found that maximum growth of callus occurred when the medium contained 15 percent coconut milk.

Maheshwari and Lal (1961) reported that in Nitsch's medium with 0.5 mg/l kinetin and 5 mg/l IAA (indole-3-acetic acid), callus was formed from the pedicel of ovaries of Iberis amara L. Bristow (1962) was successful in obtaining callus from the leaves, stem sections and apogamous buds of Pteris cretica L. He found that sugar and auxin were necessary for the formation of callus. He also found that this callus could be maintained on White's medium and on Moore's medium supplemented with 2 percent sugar and 1 mg/l 2,4-D.

Sommer, Bradley and Creasy (1962) reported that abundant callus was obtained from peach mesocarp only when 2 µg/ml kinetin was added or when coconut milk made up 20 percent of the volume of White's medium which had been modified by Skoog and Tsui (1948). They also reported that the growth of callus was hastened by the addition of 10 µg/ml naphthalene acetic acid to the medium.

Nickell (1964) reported callus formation from internodal parenchyma tissue of several sugarcane varieties using White's medium with 18 percent coconut milk and 6 ppm 2,4-D. Mohan Ram and Steward (1964) obtained callus from explants of the pericarp of banana fruits and found that this callus was capable of unlimited growth in a medium of

White's mineral salts, 2 percent sucrose, 0.5 percent agar, and supplemented with coconut milk plus 2,4-D, BTOA (benzothiazole-2-oxyacetic), coumarin, 2,3,6-trichlorophenylacetic acid and other substituted phenylacetic acids, kinetin, adenine sulphate or IAA.

Chen and Galston (1965) obtained callus from pith excised from Pelargonium stem by transferring the pith tissue to a semi-solid medium containing auxin and cytokinin. Earle (1965) reported obtaining callus from cultures of roots of Convolvulus arvensis L. by adding 0.22 mg/l 2,4-D, 40.4 mg/l adenine sulfate and 0.22 mg/l kinetin to the basal medium.

Steeves, Gabriel and Steeves (1967) reported success in obtaining callus from the excised leaves of Helianthus annuus L. and Nicotiana tabacum L., and showed that Heller's solution of mineral salts with 2 percent sucrose, a mixture of ten B vitamins, 15 percent autoclaved coconut milk and acid casein hydrolysate enhanced callus growth.

Recently Van't Hof and McMillan (1969) observed the formation of two different callus tissues, one composed of diploid and the other of a mixture of diploid and polyploid cells by incorporation of kinetin in the culture medium. These callus tissues were obtained by culturing 1 mm pea root segments and were the result of gross morphological changes associated with the anatomical location of the proliferative cells.

2. Media with supplements other than auxin and cytokinin

Auxin and cytokinin have been used as supplements to the basal medium for callus growth by many workers, however, some organic and inorganic substances were also thought to possess the callus growth

stimulating abilities. Morel and Wetmore (1951a) stated that vigorous growth of fern callus tissue could be obtained from the spores of Osmunda cinnamomea L. by treating them with vitamins such as thiamin, inositol, pyridoxin, calcium d-pantothenate, biotin, nicotinic acid in Knudson's medium.

Barker (1953) using White's medium, modified by the addition of increased quantities of the organic supplement, such as thiamin and myo-inositol, obtained callus from medullary ray tissue excised adjacent to the pith of a 50-year old linden tree, and reported that the callus originating from the tissue of the medullary sheath, contained nucleate cells which appeared meristematic.

LaRue (1947) reported callus formation from corn endosperm. Straus and LaRue (1954) subsequently investigated this in detail and found that these cultures could be maintained with unlimited growth by adding 2 percent sucrose and 0.5 percent filtered yeast extract to the basal medium. Mascarenhas, Sayagaver and Jagannathan (1965) reported callus formation from segments of maize seedlings when using White's medium containing ferric citrate instead of ferric sulphate.

White (1967) found that callus tissues were formed from explants, consisting only of xylem-cambium-phloem tissues without pith or cortex of mature spruce trees, which were placed on nutrient agar.

Organized growth of different plants from callus tissues

After callus tissues were successfully obtained from many species, organized growth from the callus tissues seemed to be the next goal of the workers. It was hoped that this work would be extensively useful

in fundamental and applied research in the fields of biology, physiology, genetics, etc. Skoog (1944) reported that tissues obtained by sub-culturing a piece of callus originally from pith tissue of a tobacco hybrid, which had been established by White in 1939 could produce stems, leaves, and roots. He also reported that these organs, arising directly from undifferentiated tissues, were capable of reverting to undifferentiated growth.

Morel and Wetmore (1951b), working with tissue cultures of Amorphophallus rivieri Dur. (Hydrosome), reported that they could obtain buds and roots from the callus tissues of this material. Skoog and Miller (1957) stated that initiation of buds and roots could be induced from tobacco pith callus by the interaction of kinetin and IAA. Tulecke (1957) reported that callus was formed from the pollen of Ginkgo biloba L. grown in a mixture of White's medium and Nitsch's medium. He also found that this callus was made up of meristematic centers and parenchyma cell derivatives.

Steward, Mapes and Mears (1958) reported that they obtained roots and subsequently buds from callus tissues derived from freely suspended cells which had been obtained from explants of carrot phloem tissue placed in solution culture. Subsequently shoots and roots developed, and the plantlets matured and even flowered. These plants were comparable to field-grown plants in the development of typical orange colored and swollen roots and could be successfully transplanted to soil.

Schroeder, Kay, and Davis (1962) observed that the callus derived from the pericarp of avocado developed roots with characteristic root

caps and a tetrarch arrangement of the vascular bundles. Wimber (1963) succeeded in propagating Cymbidium clones from callus tissues which were derived from shoot tissue of parent clones. Yamada (1963) in culturing Lilium anthers obtained callus from the cut ends of the anthers. He later observed the development of shoots and roots from the callus when transferred to media containing either adenine or NAA (naphthalene-acetic acid).

Guha and Maheshwari (1964) reported that they obtained embryo-like bodies from callus tissues derived from excised anthers of Datura collected at the mature pollen grain stage. These embryos had a distinct root-shoot axis and the super numerary cotyledons developed into seedlings. Raghavan and Torrey (1964) reported that in a medium containing vitamins and kinetin, embryos of Capsella bursa-pastoris Medic. formed callus which subsequently differentiated leaves and secondary roots.

Konar and Natarja (1964, 1965) obtained callus from the excised floral buds of Ranunculus sceleratus L. by using White's medium with coconut milk or coconut milk and IAA. They also found that this callus developed embryoids which ranged from zygote-like to mature embryo-like structures. On isolation and culture, these produced shoots and roots, and then developed into plants. Again, Konar and Sett (quoted in Mohan Ram and Wadhi, 1964) reported that when flower buds of Phlox drummondii L. were cultured on White's medium with coconut milk at a stage in which sepal, petal and stamen primordia had differentiated, the initial callus appeared at the cut ends, and later extended to cover the entire flower buds. From such callus tissues a number of roots and usually a single shoot originated.

Chen and Galston (1967) in their experiments with Pelargonium callus showed that they could obtain shoots and roots by keeping the callus tissues in semi-solid medium for prolonged periods. Carter, Yamada and Takahashi (1967) obtained roots and shoots from callus tissues of Avena sativa var. Victory cultured on Linsmaier-Skoog's medium supplemented with IAA, kinetin and 2,4-D.

Wilmer and Hellendoorn (1968) in culturing Asparagus officinalis L. obtained not only callus tissues from hypocotyls of this material in Linsmaier-Skoog's medium containing 2,4-D and kinetin, but also shoots and roots from callus tissues when 2,4-D and kinetin were omitted from the Linsmaier-Skoog's medium.

Heinz and Mee (1969) reported that callus tissues were obtained from parenchyma tissue of shoot apices, leaves, and inflorescences of Saccharum species by using Murashige-Skoog's medium supplemented with coconut milk, 2,4-D, thiamine hydrochloride, myo-inositol. They also reported that when these callus tissues were transferred to the same medium but without the addition of 2,4-D, plantlets were obtained from the tissues. Urata (unpublished) observed callus tissues from the inflorescences of grasses and subsequently observed plants from these tissues by using Linsmaier-Skoog's medium with the coconut milk and 2,4-D additives of Steward and Caplin (1951).

Pillai and Hildebrandt (1969) reported that they obtained shoots and roots from callus tissues of Geranium stem tips and internode pith tissues, and stated that these callus tissues grew well in synthetic media with or without coconut milk.

The successful production of haploid plants from callus was

reported by Niizeki and Oono (1968). They obtained the haploid callus and plants from anther culture in Oryza sativa L. on Blaydes medium supplemented with various growth substances such as IAA, kinetin, 2,4-D, adenine sulfate and yeast extract singly or in combination. Nitsch and Nitsch (1969) reported that hundreds of haploid plants of various species of Nicotiana could be raised from the callus of pollen grains. They also reported that the plants would mature and flower profusely in the simple medium. These plants did not set seed, because they were haploid. These methods lead directly to homozygosity in one generation, a technique which may be useful for plant breeding under certain conditions.

MATERIALS AND METHODS

Materials

Plant materials used in this experiment for callus formation and for organized growth from callus were as follows:

Avena sativa L.

Cenchrus echinatus L.

Chloris barbata (L.) Swartz

Chloris divaricata R. Br.

Chloris radiata (L.) Swartz

Cynodon dactylon (L.) Pers.

Cynodon dactylon var. Florida No-mow

Cynodon dactylon var. Tif lawn

Digitaria decumbens Stent. (triploid)

Digitaria decumbens Stent. (hexaploid)

Digitaria pentzii Stent. var. A24

Eleusine indica (L.) Gaertn.

Eragrostis amabilis (L.) Wight et Arn. ex Nees.

Eragrostis pectinacea (Michx.)

Oryza sativa L.

Paspalum conjugatum Bergius.

Sacciolepis contracta Wight and Arn.

Setaria verticillata (L.) Beauv.

Triticum aestivum X *Secale cereale* (*Triticale*)

Triticum aestivum L.

A number of weed species, such as Chloris barbata, Chloris radiata, and Eleusine indica, as well as the lawn grass Cynodon dactylon were collected from the campus of the University of Hawaii.

Seeds of Oryza sativa and Triticale were obtained from Dr. Shosuke Goto of the University of Hawaii. Seeds of Avena sativa and Triticum aestivum were obtained from the Institute of Agriculture of the University of Minnesota.

1. Media

Linsmaier-Skoog's medium was the basic medium used with or without coconut milk and 2,4-D for culturing the grass inflorescences. The two growth-promoting substances, coconut milk and 2,4-D were first recommended by Steward and Caplin (1951) and are now considered as cytokinin and auxin, respectively (Letham, 1969). Linsmaier-Skoog's medium with or without coconut milk and 2,4-D was also used as a solid medium with one percent agar or as a liquid medium without agar. The composition of Linsmaier-Skoog's medium is given in Table I. The four media which were used in the experiment and based on Linsmaier-Skoog's medium were prepared as follows:

a. SC Solid Medium: SC solid medium was prepared with Linsmaier-Skoog's medium plus 10 percent coconut milk by volume and 6 ppm 2,4-D. The designation SC was derived from the names of Steward and Caplin (1951), since they first recommended the use of coconut milk and 2,4-D together with the inorganic salts and other organic nutrients.

b. CM Solid Medium: CM solid medium was prepared with Linsmaier-Skoog's medium plus 10 percent coconut milk (CM) by volume.

TABLE I. COMPOSITION OF LINSMAIER-SKOOG'S MEDIUM

A. Mineral salts (after Linsmaier and Skoog, 1965) (Inorganic constituents)			
Major elements		Minor elements	
Salts	mg/l	Salts	mg/l
NH_4NO_3	1650	H_3BO_3	6.2
KNO_3	1900	$MnSO_4 \cdot 4H_2O$	22.3
$CaCl_2 \cdot 2H_2O$	440	$ZnSO_4 \cdot 4H_2O$	8.6
$MgSO_4 \cdot 7H_2O$	370	KI	0.83
KH_2PO_4	170	$Na_2MoO_4 \cdot 2H_2O$	0.25
Na_2EDTA*	37.3	$CuSO_4 \cdot 5H_2O$	0.025
$Fe_2SO_4 \cdot 7H_2O$	27.8	$CoCl_2 \cdot 6H_2O$	0.025
B. Organic constituents			
Sucrose	20 g/l	Thiamine	1 mg/l
Agar	10 g/l	myo-Inositol	100 mg/l

*NaEDTA = Disodium Ethylenedinitrilotetraacetate or Disodium Ethylenediaminetetraacetate.

No 2,4-D was added in this medium.

c. 2,4-D Solid Medium: 2,4-D solid medium was prepared from the Linsmaier-Skoog's medium to which 6 ppm 2,4-D was added. Coconut milk was omitted from this medium.

d. SC Liquid Medium: SC liquid medium was the only medium prepared without agar in this experiment. It consisted of Linsmaier-Skoog's medium plus 10 percent coconut milk by volume and 6 ppm 2,4-D from an alcohol solution.

2. Methods of culture

Success of tissue culture was closely related to the medium and conditions of nurture. Three techniques, flasks and test tubes with solid media, rotator with liquid media and auxophyton with liquid medium, were used in the course of this study for inducing callus from inflorescences of tested materials and for inducing plants from the callus. The last two techniques which involved liquid media were tested to compare callus formation at the different speeds of rotation of the devices.

a. Flask and test tube with solid media technique: In tests with solid media, 50 ml Erlenmeyer flasks and 25 X 100 mm Pyrex test tubes were employed. A bit of inflorescence about 1 1/2-2 cm long was transferred aseptically to the flask or test tube. This method was used in the majority of the species for callus formation and for organized growth of callus. SC solid medium and 2,4-D solid medium were used for inducing callus from inflorescence. If the small piece of

tissue formed callus which later became green it was transferred to another medium for organized growth. CM solid medium was used for this purpose.

Although solid medium was used for the bulk of the studies, several mechanical devices for growing explants in liquid medium were also evaluated against the method with solid media. The two techniques used were described below.

b. Rotator with liquid medium technique: The rotator technique was introduced by Gey and Gey (1936) for the culture of animal tissues in liquid media. It was a method in which the test tubes were mounted on a disk which was revolved around a shaft tilted at about 15 degrees from the horizontal, so that each test tube was rotated around its long axis (Figure 1). In animal tissue culture, the cultures were fixed to the sides of the test tube by blood clots so that the culture was alternately immersed and exposed to the air. The animal tissue cultures were usually run at 1 RPM or 1/5 RPM. The rotator speed in this experiment was 40 RPM. The use of a faster rotator was to compensate for the lack of means to fix the cultures to the sides of the tubes. Pyrex test tubes which were 25 X 150 mm and contained 15 ml liquid medium were used.

c. Auxophyton with liquid medium technique: The auxophyton technique, devised by Steward, Caplin, and Millar (1952), was used with modified apparatus in this laboratory. It involved the use of specially designed vessels with air pockets on the side. There were five sets of clamps for holding the vessels. These five sets of clamps were connected

to the main shaft in groups of twelve. The vessels were held in position, so that the pockets rotated perpendicularly to the shaft. A gear motor and speed reducer was used to maintain the shaft speed at 1 RPM. Vessels which were 500 ml volume and contained 100 ml liquid medium were used in this technique (Figure 2).

The rotator and auxophyton techniques were used only with the inflorescences of Chloris barbata and Cynodon dactylon for comparison of callus formation. The rest of the species were cultured in nutrient agar for inducing callus formation.

3. Procedures

Procedures for culturing the inflorescences involved the following steps:

a. Materials were collected at an early flowering stage when the inflorescence was still enclosed in a leaf.

b. The inflorescences enclosed in the leaves were then dipped in 95 percent ethyl alcohol for a few seconds.

c. They were placed in PMA (phenyl mercuric acetate 1:4,000) solution for 10-15 minutes (Wismer, 1960).

d. The leaves were peeled off the inflorescence with a pair of forceps in a dust-free room and a small piece of tissue was removed from the base of the inflorescence and put in either SC solid medium, 2,4-D solid medium or SC liquid medium for inducing callus formation. If this piece of tissue formed an initial callus which turned green, it was transferred to CM solid medium and CM liquid medium to induce organized growth.

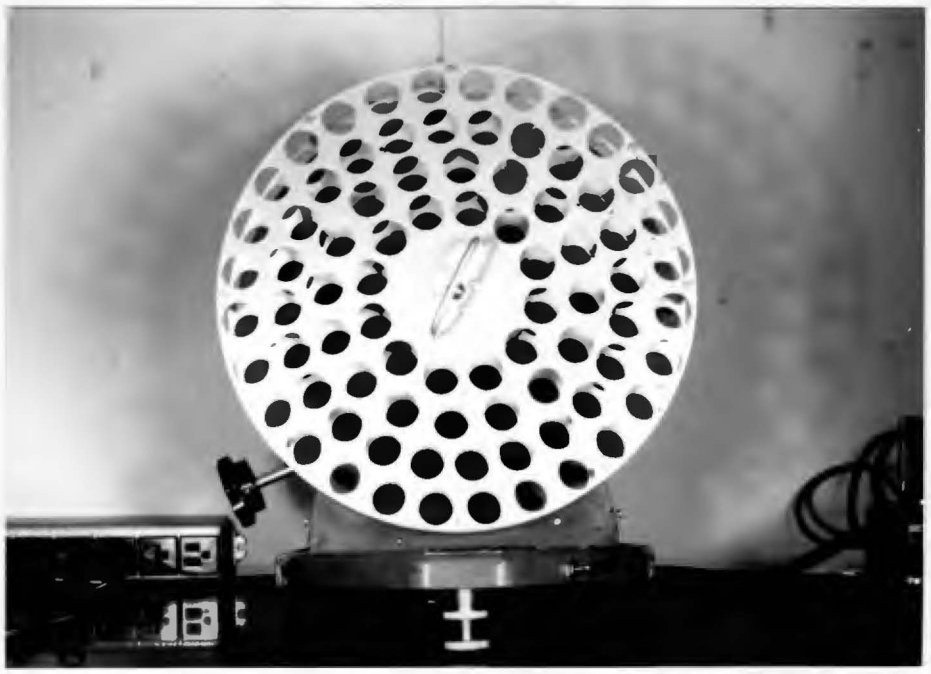

FIGURE 1. THE ROTATOR

FIGURE 2. THE AUXOPHYTON

4. Microtechnique for anatomical studies

Cynodon dactylon var. Florida No-mow and Chloris barbata were chosen for these studies because the former did not form plants from callus tissues grown on solid medium, while the latter did. Inflorescences from both species were first cultured in the SC solid medium and then were separately subcultured in SC and CM solid media as soon as the callus was formed. The subcultures in SC and CM solid media were fixed at 4, 8, 16, and 24 days after the transfer. They were then dehydrated and embedded in order to study their anatomical changes at different intervals after transfer.

Materials were fixed in Craf fixative (Randolph's modified Navashin Fluid) for 12-24 hours, then transferred directly to 70 percent ethyl alcohol or to FAA (Formalin 5 cc, acetic acid 5 cc, and 70 percent ethyl alcohol 90 cc). The alcohol was changed 3 or 4 times at 15-minute intervals then the specimen was dehydrated and embedded in paraffin by the tertiary butyl alcohol method (Johansen, 1940). Sectioning and staining were performed on the embedded materials. Three stains, Delafields' hematoxylin, Heidenhain's iron hematoxylin, and Safranin fast green were used in order to determine which would give the best results. Johansen's method (1940) of sample preparation was used for all three staining procedures.

RESULTS AND DISCUSSION

Observations of callus formation in SC solid medium and organized growth of callus in CM solid medium

The inflorescences of 17 species and 13 varieties of grasses were used in this study. At least 7 inflorescences of each species or variety, were first transferred to SC solid medium for callus formation and later transferred to CM solid medium for organized growth when the initial callus tissues derived from the inflorescences became green. In previous work, it was shown that green callus tissues were usually necessary for the formation of plants (Urata, unpublished). This procedure was followed in this experiment in order to assess the effectiveness of the SC and CM solid media in inducing callus from inflorescences and formation of plants from callus since this method had also been successfully worked out in Saccharum species by Heinz and Mee (1969).

Inflorescences of the tested materials, when transferred to SC solid medium, usually formed callus tissues after several days. The callus tissues were found to be of two different types; one type formed a single large entity, as the callus tissues of Cynodon dactylon var. Florida No-mow, Triticum aestivum X Secale cereale (Triticale) and Triticum aestivum (Figure 3), and the other type formed numerous small nodules, as the callus tissues of Chloris barbata, Chloris divaricata, Chloris radiata, Cynodon dactylon, Cynodon dactylon var. Tif lawn, Digitaria decumbens (triploid), Digitaria decumbens (hexaploid), Digitaria pentzii var. A 24, Eleusine indica, Eragrostis amabilis, Eragrostis pectinacea, Oryza sativa, Paspalum conjugatum, Sacciolepis contracta, and Setaria verticillata (Figure 4).

FIGURE 3. EXAMPLE OF CALLUS TISSUES FORMING AS A SINGLE LARGE MASS FROM CYNODON DACTYLON VAR. FLORIDA NO-MOW.

FIGURE 4. EXAMPLE OF CALLUS TISSUES FORMING NUMEROUS SMALL NODULES FROM ERAGROSTIS PECTINACEA.

The shape or form of the callus tissues found in the plant materials, however, was found to be unrelated to callus growth and the development of organized growth later since examples of organized growth were found in both types.

After the callus tissues were formed in SC solid medium, they usually and gradually became green. This sometimes occurred only in limited parts of the callus tissues. At that time, they were transferred to CM solid medium for differentiation into organized growth. Callus tissues were found to differ in time of formation and in organ initiation. Results of these experiments are summarized in Table II and are discussed below.

Avena sativa

No callus formation was observed and the explant died soon after it was transferred to the medium.

Cenchrus echinatus

Five days after being placed in the medium inflorescences started to turn brown and gradually died.

Chloris barbata

Callus tissues were observed on the 6th day, green callus tissues on the 22nd day and plants on the 55th day. The plants from callus tissues were transplanted in soil and they developed well (Figure 5). This species was also found to form albino callus tissues and albino plants. These are described in a later section.

TABLE II. OBSERVATIONS ON CALLUS FORMATION AND ORGANIZED GROWTH OF EACH GRASS SPECIES IN DIFFERENT MEDIA

Species	Media[a]				Days for callus formation	Type of callus[b]			Type of organ		Whole plant
	SC-solid	2,4-D	CM	SC-liquid		Initial	Green	Albino	Root	Shoot	
Avena sativa	+*	+*	-*	-*		-	-	-	-	-	-
Cenchrus echinatus	+*	-*	-*	-*	-	-	-	-	-	-	-
Chloris barbata	+*	+*	+*	+*	6	+	+	+	+	+	+
Chloris divaricata	+*	+*	-*	-*	13	+	-	-	-	-	-
Chloris radiata	+*	-*	-*	-*	8	+	+	-	-	-	-
Cynodon dactylon	+*	+*	-*	+*	12	+	-	-	-	-	-
Cynodon dactylon var. Florida No-mow	+*	+*	+*	-*	7	+	-	-	-	-	-
Cynodon dactylon var. Tif lawn	+*	+*	-*	-*	7	+	-	-	-	-	-
Digitaria decumbens (triploid)	+*	-*	-*	-*	4	+	-	-	-	-	-
Digitaria decumbens (hexaploid)	+*	-*	+*	-*	5	+	+	-	+	+	+
Digitaria pentzii var. A24	+*	-*	+*	-*	5	+	+	-	+	+	+
Eleusine indica	+*	+*	-*	-*	12	+	-	-	-	-	-
Eragrostis amabilis	+*	-*	+*	-*	6	+	+	-	+	+	+
Eragrostis pectinacea	+*	-*	+*	-*	7	+	+	-	-	+	-
Oryza sativa	+*	-*	-*	-*	20	+	-	-	-	-	-
Paspalum conjugatum	+*	-*	+*	-*	13	+	+	-	-	-	-
Sacciolepis contracta	+*	-*	-*	-*	9	+	-	-	-	-	-
Setaria verticillata	+*	-*	+*	-*	12	+	+	-	-	-	-
Triticale	+*	-*	+*	-*	10	+	-	-	+	-	-
Triticum aestivum	+*	-*	-*	-*	16	+	-	-	-	-	-

[a] +*: Inflorescences cultured in medium. -*: No inflorescence cultured in medium.

[b] + : growth - : no growth

FIGURE 5. PLANTS FROM CALLUS TISSUES OF CHLORIS BARBATA.

Chloris divaricata

Callus tissues of this species were observed on the 13th day. It then grew slowly and then gradually died. No green callus was observed in this species.

Chloris radiata

Callus tissues were observed on the 8th day, green callus tissues were observed on the 15th day, but they failed to grow shoots or roots.

Cynodon dactylon

Callus tissues were observed on the 12th day, and later they gradually became brown and died. Green callus tissue or organ initiation of this species was not observed.

Cynodon dactylon var. Florida No-mow

Callus tissues were observed on the 7th day. The callus tissues had a tremendously fast growth rate. It also found that these callus tissues could be maintained by periodic transfers. Thus, they were thought to have the capacity for unlimited growth. Green callus tissues and organ formation did not occur in this variety.

Cynodon dactylon var. Tif lawn

Callus tissues were observed on the 7th day, but callus turned brown and gradually died.

Digitaria decumbens (triploid)

Callus tissues which were observed on the 4th day gradually died. However, this clone had been propagated by callus tissues in this laboratory.

Digitaria decumbens (hexaploid)

Callus tissues were observed on the 5th day, green callus tissues on the 18th day and plants on the 42nd day. This clone was more adaptable in SC solid medium than the triploid clone of the same species in this study.

Digitaria pentzii var. A 24

This species is in the same genus with Digitaria decumbens (triploid and hexaploid). Callus tissues and green callus tissues were observed on the 5th day and the 25th day, respectively, and on the 32nd day, plants started to form. Urata (unpublished) was successful in deriving plants of this variety from callus tissues.

Eleusine indica

Callus tissues of this species grew very slowly after they were observed on the 12th day. Green callus tissue or organ formation, however, was not observed.

Eragrostis amabilis

Callus tissues were observed on the 6th day after the inflorescences were transferred to the medium. Green callus tissues were observed on the 27th day, and plants on the 55th day. Plants of this species which were transplanted in soil grew well (Figure 6).

Eragrostis pectinacea

Callus tissues were observed on the 7th day. These tissues started to turn green on the 57th day. Some shoots were found growing from the green callus tissues on the 74th day, but they gradually died. Growth of shoots appeared to be inhibited for some unknown reason.

FIGURE 6. PLANTS FROM CALLUS TISSUES OF ERAGROSTIS AMABILIS.

Oryza sativa

Callus tissues of this species were observed on the 21st day. However, no green callus or organ developed. This species had been cultured for more than one year through several passages and still appeared in good condition at the time of writing this paper. The growth rate of the callus tissues was very slow.

Paspalum conjugatum

Callus tissues of this species were observed on the 13th day and became green on the 38th day, then they gradually died.

Sacciolepis contracta

Callus tissues which were observed on the 9th day gradually died.

Setaria verticillata

Callus tissues were observed on the 12th day, and turned green on the 28th day after transfer. This green callus tissue however did not form any shoot or root and gradually became brown and died.

Triticum aestivum X Secale cereale (Triticale)

This species was found to grow only roots after the callus tissues were observed on the 10th day. No green callus tissue was observed. Subcultures of this species could be maintained for several transfers. Roots derived from initial callus tissues occurred in all of the subcultures.

Triticum aestivum

The callus tissues of this species were observed on the 16th day. Callus growth was slow, but they survived for nearly three months.

Neither green callus nor organ formation was observed in this species.

Observations on callus formation of several species in SC solid medium and 2,4-D solid medium

Several grass species had been reported to form callus tissues very well in SC solid medium (Carter, Yamada and Takahashi, 1967; Urata, unpublished). SC solid medium, as explained in a previous section, was the Linsmaier-Skoog's medium supplemented with coconut milk and 2,4-D. The 2,4-D solid medium was the Linsmaier-Skoog's medium supplemented only with 2,4-D. In the following experiment, several grass species were picked at random to test the effectiveness of these two different media in inducing callus formation. The species were Avena sativa, Chloris barbata, Chloris divaricata, Cynodon dactylon, Cynodon dactylon var. Florida No-mow, Cynodon dactylon var. Tif lawn, and Eleusine indica. Five inflorescences of every species were put into each medium. Results of this experiment are presented in Table III and are discussed below.

Avena sativa

Callus formation did not occur in either SC solid medium or 2,4-D solid medium. Inflorescences became brown about one week after being transferred.

Chloris barbata

Callus tissues were observed in both media. However, the callus tissues in 2,4-D solid medium were whiter and the growth rate was slower. The callus tissues in 2,4-D solid medium, therefore, did not grow as well as those in SC solid medium even though the time of transfer was the same.

TABLE III. CALLUS FORMATION OF SPECIES CULTURED IN SC SOLID MEDIUM AND 2,4-D SOLID MEDIUM

Species	SC Solid Medium[a]	2,4-D Solid Medium[a]
Avena sativa	-	-
Chloris barbata	+*	+
Chloris divaricata	+*	+
Cynodon dactylon	+	+*
Cynodon dactylon var. Florida No-mow	+*	+
Cynodon dactylon var. Tif lawn	+*	+
Eleusine indica	+*	+

[a] + : callus growth
+* : better callus growth
- : no callus growth

Chloris divaricata

Callus formation occurred in both media, but callus tissues grew less frequently and more slowly in the 2,4-D solid medium when compared with those in the SC solid medium.

Cynodon dactylon

This species behaved differently from the other species in that callus tissues were found to grow better in 2,4-D solid medium. More callus tissues were formed in 2,4-D solid medium than in SC solid medium.

Cynodon dactylon var. Florida No-mow

The callus tissues of this variety were found to grow profusely in SC solid medium when compared with those grown in 2,4-D solid medium in which callus initiated less frequently and more slowly.

Cynodon dactylon var. Tif lawn

More callus tissue formation took place in SC solid medium than in 2,4-D solid medium. However, the callus tissues in both media only survived about one week.

Eleusine indica

Callus tissues of this species grew better in SC solid medium than in 2,4-D solid medium, but callus tissues in both media only lived for about ten days.

Discussion of behavior of various species grown in vitro

Generally, callus tissues formed from the inflorescences, and later these tissues turned green before they initiated shoots or roots. However, the behavior of callus *in vitro* varied, depending upon the species. For example, *Triticale* formed roots only and these roots were formed directly from callus tissues which had not turned green. *Chloris barbata* formed callus tissues as well as albino callus tissues which later developed into albino plants. *Cynodon dactylon* var. Florida No-mow and *Oryza sativa* grew callus tissues which were maintained more than one year by periodic transfers. However, these tissues did not turn green and did not form shoots or roots. *Avena sativa* and *Cenchrus echinatus* did not grow callus or survive in the medium.

Callus tissues of all species were observed to become brown when they were kept in the medium for a long time and would die unless transferred. Callus tissues which had become brown would also affect the medium if they were kept in the same medium for a long period of time. The medium was then found to be brown below the callus tissues.

Plants were usually found to be fully developed (roots and shoots) within one week after they were initiated from callus tissues. These plants grew well in the medium for a month, then they gradually turned brown and died if they were kept in the same medium too long. Callus tissues initially formed at the excised end of the inflorescences and at the base of the florets, especially at the latter. Time from explantation to callus formation was varied from four to twenty days.

Observations on callus formation in liquid medium in the rotator and the auxophyton

Although the agar method, using solid medium was used for the bulk of the studies, several mechanical devices for growing explants in liquid media were also evaluated against the agar method. With species such as carrot (Steward, 1958) and Orchidaceae (Wimber, 1963) callus grown in liquid media with some type of mechanical means of aeration had been shown to give faster growth than callus grown in solid medium.

As mentioned previously, the rotator held test tubes and depended on rapid rotation (40 RPM) to aerate the liquid, while the auxophyton had a slower rotation rate (1 RPM) but depended on specially blown pockets or "nipples" which trapped and raised the callus tissues into the air during part of the rotation cycle.

These two different mechanical devices were used on two species to determine which of them would work out better for callus formation and callus growth with respect to the different speeds of rotation and the conditions of nurture. In the rotator, the tissue was usually immersed, while in the auxophyton it was usually lifted into the air for part of each cycle.

Species used were Chloris barbata and Cynodon dactylon. It was found that Chloris barbata and Cynodon dactylon formed callus tissues by the 6th day and the 8th day, respectively by the rotator technique, and by the 10th day and 13th day, respectively by the auxophyton technique after the transfer. Callus tissues from the rotator grew profusely, but the callus tissue from the auxophyton darkened and became necrotic. However, results were similar in that they did not induce green callus tissues from the initial callus tissues and also, they did not induce

plants from the callus tissues.

Albino callus tissues in Chloris barbata

Chloris barbata inflorescences were obtained from the culture maintained in the Agronomy nursery as well as from plants growing as weeds around the campus. Albino callus tissues were obtained only from clones maintained in the nursery. Albino callus segments were obtained from two separate inflorescences taken from plants at the nursery. Both albino and green initial callus tissues were similar in the early stage of development and they could not be distinguished until the callus tissues turned green or albino shoots were observed. Albino shoots developed as well in CM solid medium as did the shoots from green callus tissues which were derived from the initial callus tissues (Figure 7). However, it was found that subcultures of albino callus tissues grew rather slowly if they were transferred from CM solid medium back to SC solid medium which was usually used for inducing callus tissues.

Besides albino plants developed from the albino callus tissues, a plant with leaves generally longitudinally almost equally green and white was obtained from the albino callus tissues (Figure 8). This green and white plant was transplanted to soil and grew well. This plant was interesting because it indicated that it originally initiated from two meristematic cells, one green and one albino. However, the question as to whether the albino cells occurred from mutation or from the natural positioning of an albino tissue area near a green tissue area was moot, the latter alternative was somehow considered more

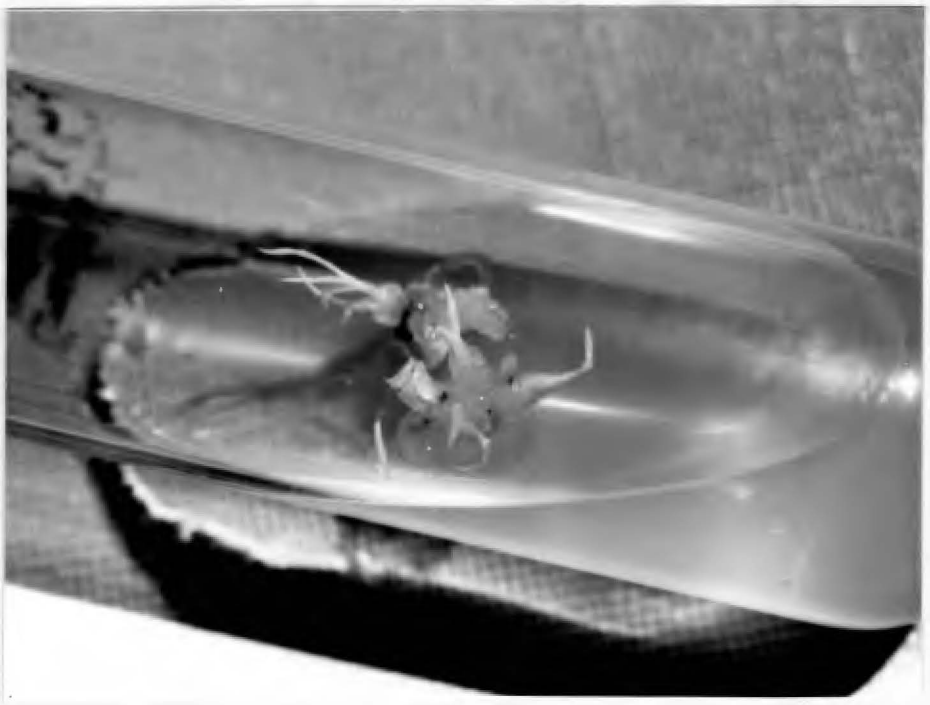

FIGURE 7. ALBINO PLANTS FROM ALBINO CALLUS TISSUES OF CHLORIS BARBATA IN CM SOLID MEDIUM.

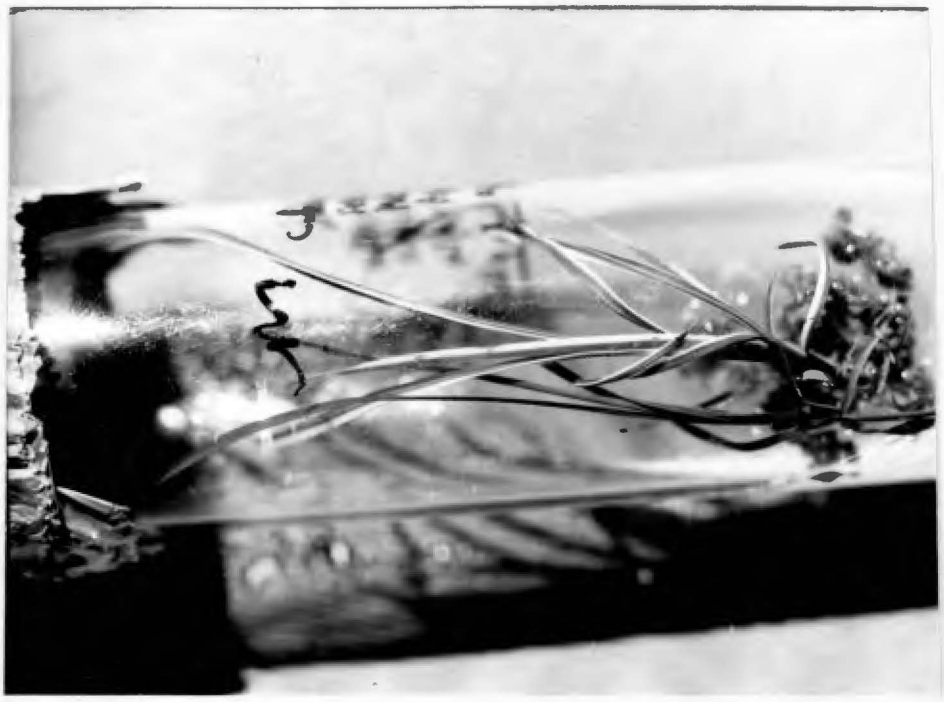

FIGURE 8. PLANTS WITH GREEN AND WHITE STRIPES FROM CALLUS TISSUES OF CHLORIS BARBATA IN CM SOLID MEDIUM.

likely. The infrequent occurrence of albino tissues in the early stages might indicate that these were mutational events. The more frequent appearance of albino mixed with green tissues that were observed as the albino tissues were transferred would then indicate a sort of contamination of albino with green tissues. Until something is known of the genetic nature of these plants, definitive answers as to the origin of these albinos are not available.

Rapidly growing callus tissues from a Cynodon dactylon var. Florida No-mow

Cynodon dactylon var. Florida No-mow was one of the lawn grasses cultivated in the Agronomy nursery. The callus tissues obtained from it had the greatest capacity for rapid growth of any of the species tested. The callus tissues of this variety grew as well as the callus tissues of other species after transfer, but, when these tissues were transferred to a new medium they grew rapidly. It was found that the callus tissues grew to almost cover the surface of the medium in the 25 X 100 mm culture tube within three weeks (Figure 9). The callus tissues of this variety have been maintained for over one year with periodic transfers. Again, the callus tissues of this variety were found to grow best only in SC solid medium when compared with the growth in 2,4-D solid medium. Coconut milk which contained cytokinin was therefore shown to be essential for the callus growth of this variety.

It was interesting to find that these rapid growing callus tissues grew very well in light as well as in the dark. These tissues seemed to use up the SC solid medium in which they were cultured (Figure 10). When the rapidly growing callus tissues were kept in the same medium

FIGURE 9. CALLUS TISSUES OF CYNODON DACTYLON VAR. FLORIDA NO-MOW IN SC SOLID MEDIUM.

FIGURE 10. CALLUS TISSUES OF CYNODON DACTYLON VAR. FLORIDA NO-MOW SHOWING THE CONSUMPTION OF SC SOLID MEDIUM BY CALLUS TISSUES.

too long, they gradually turned brown and died. However, if these brown tissues were transferred to a new medium, they would again grow as rapidly as before. This variety, therefore, has shown a capacity for growth of callus. Up to the time this paper was written, the callus tissues of this variety were still in good condition and growing rapidly.

An attempt to cause interpenetration of the callus tissues of two species

Recently Ephrussi and Weiss (1969) discussed cases in Mammalia in which cell fusion occurred between widely disparate species, for example between mouse and rat cell colonies, and even between mouse and human cell colonies (Weiss and Green, 1967). Although it was recognized that such fusions would probably be impossible in higher plants, at the present stage of technique, because of the occurrence of the cellulose cell wall, it was decided that an attempt to induce fusion should be made, using two taxonomically different callus colonies.

The first species, Chloris barbata, was much slower growing than Cynodon dactylon var. Florida No-mow. It would also form plants in CM solid medium although Cynodon dactylon var. Florida No-mow would not do so, and in fact would eventually die in such a nutrient medium.

The two types of callus tissues, both obtained from cultures growing in SC solid medium, were placed in close proximity to one another in SC solid medium. After 23 days, when the Cynodon dactylon var. Florida No-mow callus tissues had overgrown the Chloris barbata callus tissues, some of the Chloris barbata callus tissues were found to be green. At this time, these mixed cultures were wholly transferred to CM solid medium. It was noted in the handling incident to transfer,

that the Cynodon dactylon var. Florida No-mow or Chloris barbata callus tissues occasionally slipped off from each other. This was interpreted as indicating that no lasting connection had occurred between the two types of callus.

After being transferred to the CM solid medium, green callus tissues of Chloris barbata continued to grow, but the callus tissues of Cynodon dactylon var. Florida No-mow became brown and died after one week. Consequently, it was concluded that the ability of the Chloris barbata callus tissues to survive in CM solid medium was not transferred to the Cynodon dactylon var. Florida No-mow callus tissues.

Time did not permit an anatomical study of the interface between the Chloris barbata and the Cynodon dactylon var. Florida No-mow callus tissues, since this would have shown whether some cells of Cynodon dactylon var. Florida No-mow attached themselves to Chloris barbata callus tissues, and vice versa.

Anatomical studies of callus tissues of Chloris barbata and Cynodon dactylon var. Florida No-mow

Chloris barbata and Cynodon dactylon var. Florida No-mow were chosen for these studies because of their contrasting growth response in the SC solid medium and CM solid medium. Callus tissues of Cynodon dactylon var. Florida No-mow grew rapidly in SC solid medium, but not the callus tissues of Chloris barbata. The callus tissues of Chloris barbata would develop into plants in CM solid medium, but not the callus tissues of Cynodon dactylon var. Florida No-mow.

Callus tissues of Chloris barbata and Cynodon dactylon var. Florida No-mow were first obtained in SC solid medium and grown in this medium

for 24 days, then, they were transferred to SC solid and CM solid media at the same time. Samples from each culture were fixed at 4, 8, 16, and 24 days after the transfer. The fixation-staining sequence was Craf fixation, followed by storage in 70 percent ethyl alcohol, and staining in Safranin fast green. However, FAA fixation followed by staining with Delafield's hematoxylin and Craf fixation followed by Delafield's hematoxylin were also used but results were not as good as Craf fixation followed by staining with Safranin fast green.

Chloris barbata callus tissues

Cells of Chloris barbata callus tissues in SC solid medium were small and densely packed when sectioned four days after transfer. The cells in callus tissues that were cultured in CM solid medium showed essentially the same configuration (Figure 11). Eight days after transfer, the cells in callus tissues in SC solid medium were found to show signs of organized growth in which delimited meristematic regions appeared (Figure 12). Callus cells in CM solid medium, on the other hand, had a bigger meristematic area and these cells were found more highly cytoplasmic. On the 16th day, meristematic areas in SC solid medium approximating structures in early shoot development were observed. Vascular bundles were also observed in callus cells cultured in SC solid medium (Figure 13). Callus cells in CM solid medium also had these structures. On the 24th day, enlargement of meristematic area in callus tissues and tracheids which indicated xylem tissue development were found in SC solid medium (Figure 14). Callus cells in CM were more highly developed.

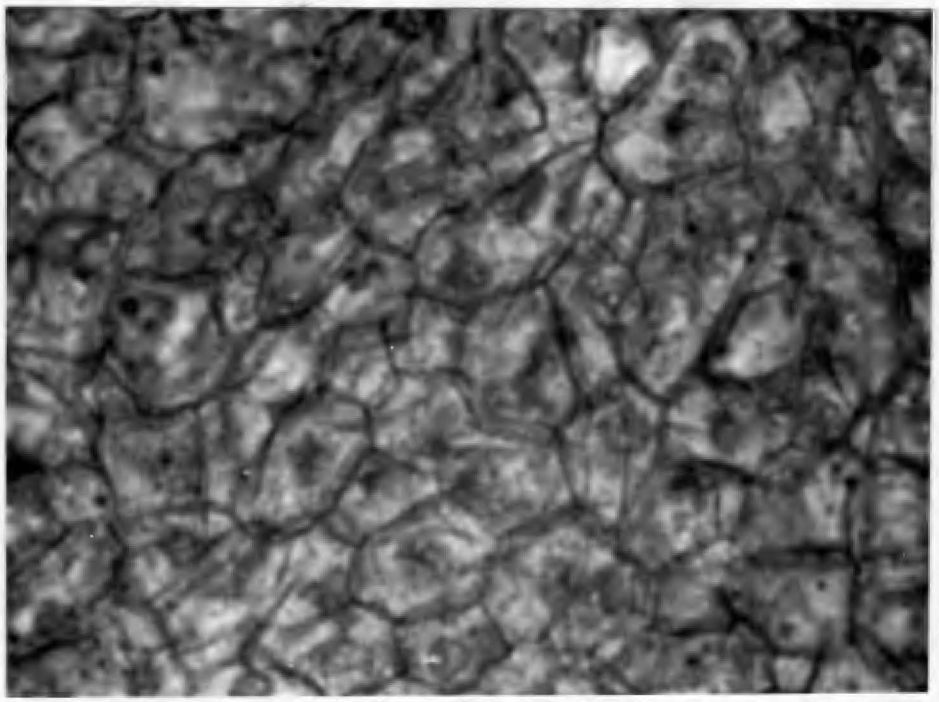

FIGURE 11. SMALL AND COMPACT CELLS OF CHLORIS BARBATA CALLUS TISSUES IN SC SOLID MEDIUM ON THE 4TH DAY AFTER TRANSFER. Ca 400X

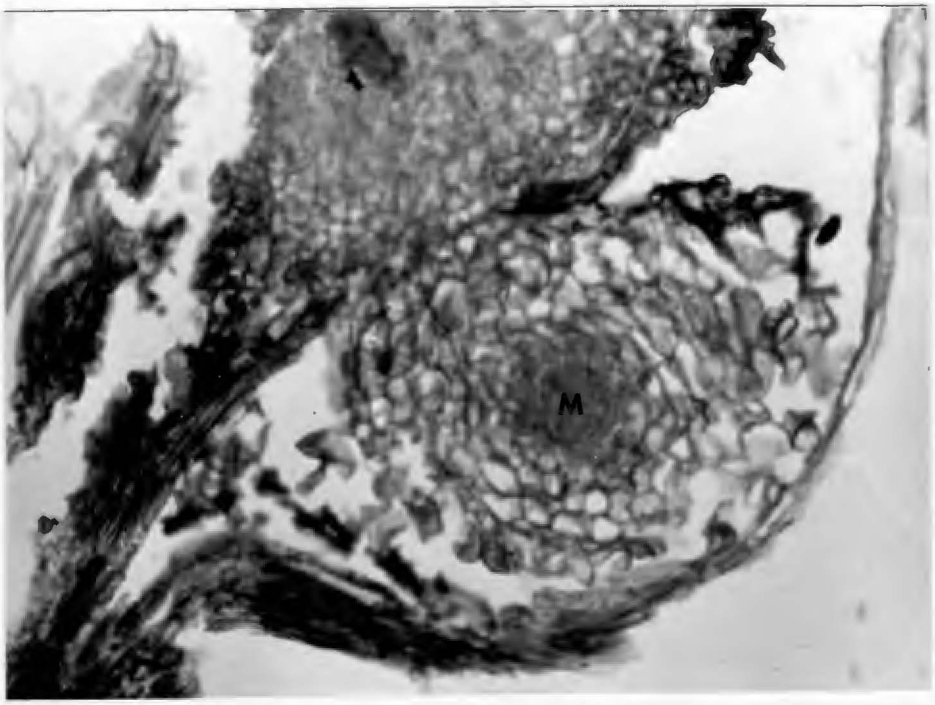

FIGURE 12. EARLY SIGN OF MERISTEMATIC AREA IN CHLORIS BARBATA CALLUS TISSUES IN SC SOLID MEDIUM ON THE 8TH DAY. Ca 100X (M: MERISTEMATIC AREA)

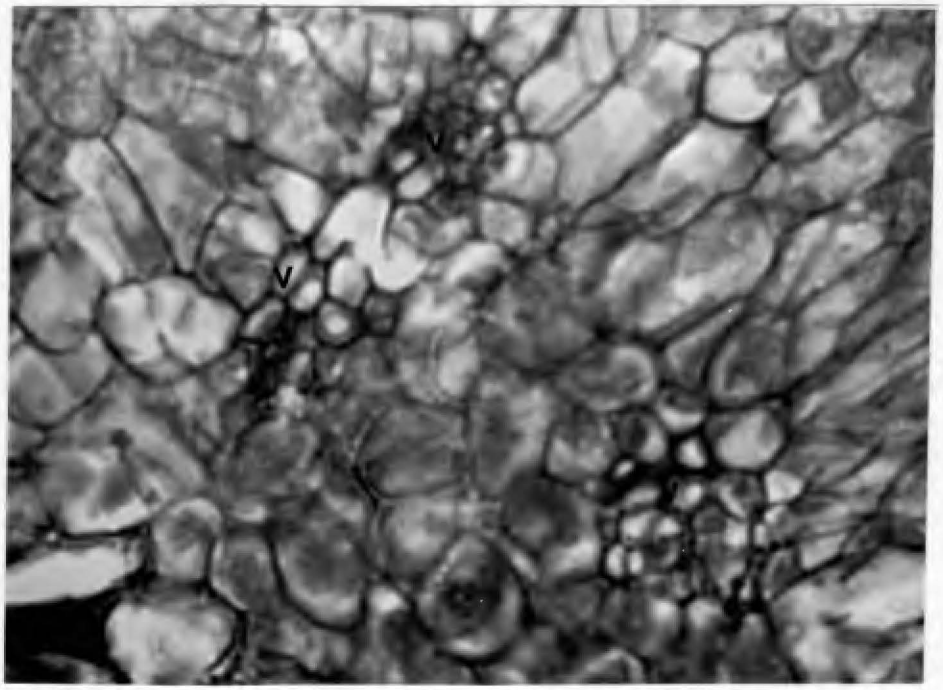

FIGURE 13. VASCULAR BUNDLES IN CHLORIS BARBATA CALLUS TISSUES IN SC SOLID MEDIUM ON THE 16TH DAY AFTER TRANSFER. Ca 400X (V: VASCULAR BUNDLE)

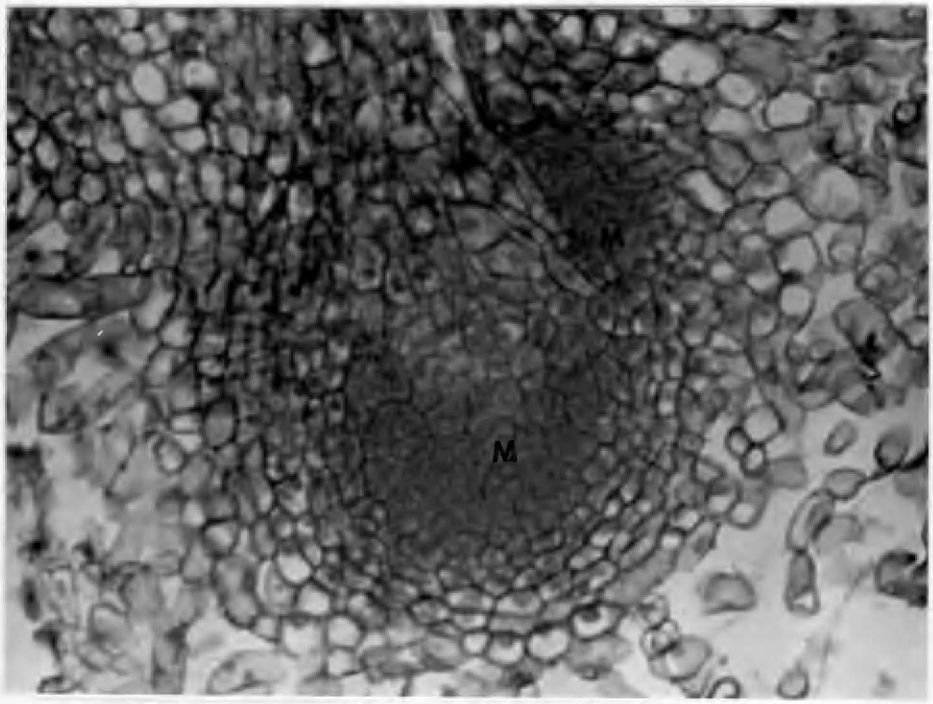

FIGURE 14. TWO MERISTEMATIC AREAS IN CHLORIS BARBATA CALLUS TISSUES IN CM SOLID MEDIUM ON THE 24TH DAY AFTER TRANSFER. Ca 100X (M: MERISTEMATIC AREA)

Cynodon dactylon var. Florida No-mow callus tissues

In the cells of Cynodon dactylon var. Florida No-mow callus tissues, anatomical change as a function of the time fixing did not occur. The callus cells were generally large and loosely packed and few nuclei were seen. Cell walls appeared to be thicker when compared with those of Chloris barbata callus tissues. The same observations were made on all callus cells for the four dates of fixation.

These observations seemed to indicate an anatomical basis for the differences in behavior of the callus tissues of the two species. The cells of Chloris barbata changed with time, at first, they were small and uniform, then they showed an organized, primordial structure in which the callus cells were richly cytoplamic and meristematic. This is probably the reason that organized growth was obtained from the callus tissues of this species. On the other hand, Cynodon dactylon var. Florida No-mow callus tissues had loosely packed parenchyma cells and with no evidence of primordial structure. This species formed only callus tissues but no shoots or roots (Figure 15). Similar results, however, had also been found in the work of Joshi (1965), Steward, Mapes and Mears (1958), and Tulecke (1957), which separately reported observations of compact parenchyma cells, loosely packed parenchyma cells and meristematic cells from the callus tissues.

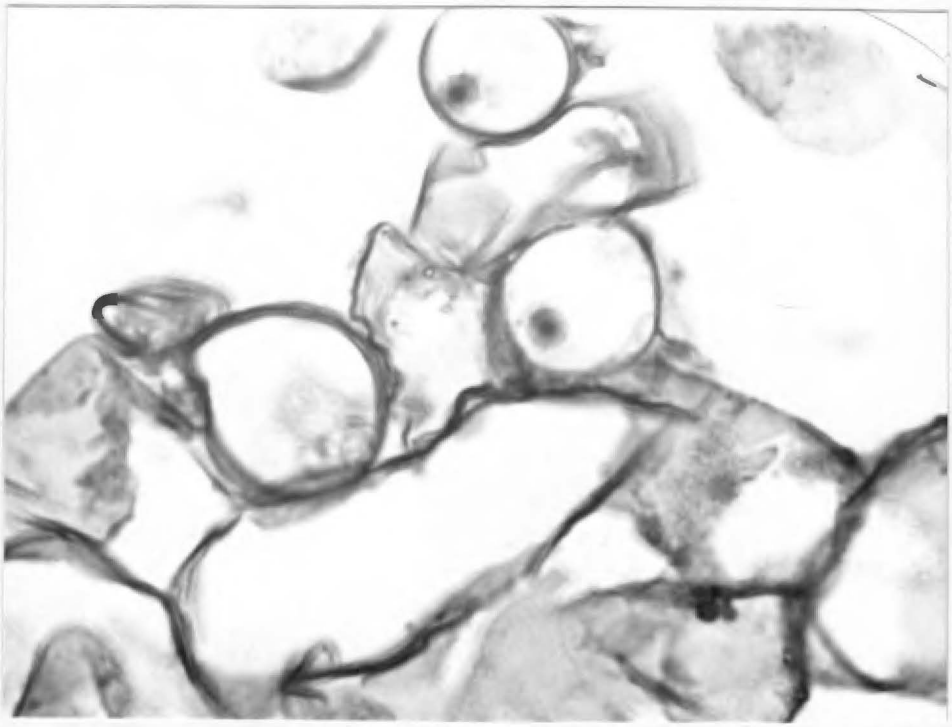

FIGURE 15. LARGE AND LOOSE CELLS OF CYNODON DACTYLON VAR. FLORIDA NO-MOW CALLUS TISSUES IN SC SOLID MEDIUM ON THE 4TH DAY AFTER TRANSFER. Ca 400X

SUMMARY AND CONCLUSIONS

1. Callus tissues were obtained from the inflorescences of 15 grass species and 3 grass varieties out of a total of 20 species by using SC solid medium, in which Linsmaier-Skoog's medium was supplemented with coconut milk (cytokinin) and 2,4-D (auxin).
2. Callus tissues of Chloris barbata, Digitaria decumbens (hexaploid), Digitaria pentzii var. A24, and Eragrostis amabilis formed plants after being transferred to CM solid medium, in which Linsmaier-Skoog's medium was supplemented only with coconut milk.
3. SC solid medium appeared to be more favorable for inducing callus formation and growth than 2,4-D solid medium for the species which were tested.
4. CM solid medium was effective for inducing callus tissues to form roots or both shoots and roots for the species which were tested.
5. The medium which was good for callus formation was not necessarily good for plant formation in the species which were tested.
6. Solid medium was found to be superior to mechanically agitated liquid medium for inducing callus formation in the experiment.
7. The rotator (40 RPM) was more effective than the auxophyton (1 RPM) in induction of callus. Both techniques were not favorable for inducing organized growth for two species, Chloris barbata and Cynodon dactylon, which were tested.
8. Callus tissues were usually initiated at the excised end of the inflorescences and at the base of the florets in all of the plant materials examined.

9. Albino and green callus tissues as well as albino and green plants were obtained from Chloris barbata.

10. A rapidly growing callus which survived numerous transfers without loss of vigor was obtained from Cynodon dactylon var. Florida No-mow, but this callus did not initiate shoots or roots.

11. An anatomical study of Cynodon dactylon var. Florida No-mow and Chloris barbata callus tissues showed that the latter species had primordial regions with meristematic cells which were lacking in the former. It was believed that these regions produce the plants in Chloris barbata.

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